IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Stephen H. Leppla et al.

Application No.: 10/088,952

Filed: March 22, 2002

For: MUTATED ANTHRAX TOXIN PROTECTIVE ANTIGEN PROTEINS THAT SPECIFICALLY TARGET CELLS CONTAINING HIGH AMOUNTS OF CELL-SURFACE METALLOPROTEINASES OR PLASMINOGEN ACTIVATOR RECEPTORS

Examiner: Brandon J. Fetterolf

Technology Center/Art Unit: 1642

Declaration of Stephen Leppla under 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Stephen H. Leppla, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both, under 18 U.S.C. § 1001, and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true and statements made on information or belief are believed to be true.

2. I hold a Ph.D. (1969) from the University of Wisconsin, and a Bachelor of Science (1963) from the California Institute of Technology. I am presently Chief, Bacterial Toxins and Therapeutics Section, at the National Institute of Allergy and Infectious Diseases ("NIAID") of the National Institutes of Health ("NIH"). My field of expertise is Bacterial Toxins and Therapeutics. I have authored over one hundred and fifty publications in the field of bacterial toxins, and am a member of the NIAID Working Group on Anthrax Vaccines; the Working Group on Recombinant Toxins, NIH Office of

Biotechnology Activities, OD, NIH; the American Society for Microbiology; and the American Academy of Microbiology. A true copy of my *Curriculum Vitae* is attached hereto as Exhibit A.

3. The present invention is directed to methods of targeting a compound to a cell using a mutant protective antigen. More particularly, the presently claimed invention is directed to methods of targeting a compound to a cell over-expressing a plasminogen activator or a plasminogen activator receptor by administering to the cell (1) a mutant protective antigen protein comprising a urokinase plasminogen activator (uPA)-recognized cleavage site in place of the native protective antigen furin-recognized cleavage site, wherein the mutant protective antigen is cleaved by the uPA; and (2) a compound comprising a lethal factor polypeptide comprising a protective antigen binding site; wherein the lethal factor polypeptide binds to cleaved protective antigen and is translocated into the cell, thereby delivering the compound to the cell. The methods can be used to kill tumor cells in vivo.

4. I am a named inventor on the above-referenced patent application. I have read and am familiar with the contents of the subject patent application. I have also read the Office Actions received from the United States Patent and Trademark Office dated December 15, 2004 and August 23, 2005. It is my understanding that the Examiner is concerned that the claimed anthrax toxin fusion proteins are obvious over U.S. Patent No. 5,677,274 ("Leppla *et al.*") in view of U.S. Patent No, 5,817,771 ("Bayley *et al.*"). Specifically, the Examiner alleges that Leppla *et al.* discloses all elements of the presently claimed methods except for a mutant protective antigen comprising a plasminogen activator-recognized cleavage site in place of the native furin-recognized cleavage site. The Examiner alleges that Bayley *et al.* disclose that a urokinase plasminogen activator cleavage site can be incorporated into a polypeptide that does not contain such a cleavage site. Based on these disclosures, the Examiner

concludes that the presently claimed methods are obvious in view of the combination of Leppla *et al.* and Bayley *et al.*

5. This declaration is provided to clarify for the Examiner that the presently claimed invention is not obvious in view of the cited references because one of skill in the art would have no reasonable expectation of success in practicing the presently claimed methods based on the combination of Leppla *et al.* and Bayley *et al.* This declaration is further provided to present data demonstrating that the mutant protective antigens of the invention can be used for *in vivo* delivery of a compound to a target cell overexpressing uPA.

6. Leppla *et al.* discloses that a compound can be delivered to a cell using a binary bacterial toxin (*i.e.*, native anthrax protective antigen or an anthrax protective antigen with an HIV-1 protease cleavage site in place of the native protective antigen cleavage site). Leppla *et al.* does not disclose or suggest a mutant protective antigen comprising a plasminogen activator-recognized cleavage site in place of the native protective antigen furin-recognized cleavage site. Bayley *et al.* does not remedy the deficiency in Leppla *et al.* Bayley *et al.* merely discloses that a uPA cleavage site can be incorporated into a polypeptide (*i.e.*, an alpha hemolysin polypeptide) that does not contain such a cleavage site.

7. Even if one of skill in the art were to combine the disclosures of Leppla *et al.*, and Bayley *et al.* there would be no reasonable expectation of success in being able to practice the presently claimed methods. The present invention is the first demonstration that a mutant protective antigen can be used to deliver a compound to a cell overexpressing a uPA. Binding of a protease to its cleavage site and subsequent proteolytic cleavage is dependent on the three dimensional structure of the proteins. One of skill in the art would not have expected that the uPA overexpressed on the surface of a target cell and the uPA cleavage site on the mutant protective antigen would have come into contact with each other. For example, the uPA cleavage site in the mutant PA might

not be positioned at an appropriate distance from the cell membrane to contact the uPA on the surface of the target cell. Accordingly, there would be no cleavage of the mutant protective antigen by the uPA or delivery of a compound to the target cell.

8. This declaration also presents experiments which demonstrate that the mutant protective antigens of the presently claimed invention are particularly effective for delivering a compound to target cells. Specifically, the experiments demonstrate that the claimed mutant protective antigens can deliver a compound to tumors overexpressing uPA when administered to tumor-bearing mice. These experiments are set forth in Rono *et al.*, *Mol Cancer Ther.* 5(1):89-96 (2006) (copy enclosed as Exhibit B) and unequivocally demonstrate that the mutant protective antigens of the invention can be used for *in vivo* delivery of a compound to a target cell overexpressing uPA. Mutant protective antigens comprising a uPA cleavage site substituted for the native furin cleavage site and compounds comprising a lethal factor polypeptide comprising a protective antigen binding site were systemically administered to mice bearing one of the following types of tumors: B16 melanoma, T241 fibrosarcoma, or Lewis lung carcinoma, all of which overexpress uPA. Administration of the mutant protective antigen and lethal factor polypeptides led to significant tumor growth inhibition, demonstrating that the mutant anthrax protective antigens are cleaved by the uPA expressed by the tumor cells and deliver a compound, *i.e.*, lethal factor, to the cells.

9. In conclusion, it is my scientific opinion that prior to the present invention, one of skill in the art would not have a reasonable expectation of success in practicing the presently claimed methods in view of the teachings of Leppla *et al.* and Bayley *et al.*. The present application provides the first evidence that a mutant protective antigen in which a uPA cleavage site had been substituted for the native furin-recognized cleavage site could be used to deliver a compound to a cell overexpressing uPA. As

demonstrated by the experiments set forth above, the mutant protective antigens of the invention are particularly effective for *in vivo* delivery of a compound to a target cell.

Dated: 22 Feb 2006 By: _____
Stephen H. Leppla, Ph.D.

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CURRICULUM VITAE

Stephen H. Leppla, Ph.D.

EDUCATION

1959-63 B.S. Biology, California Institute of Technology

1963-69 Ph.D. Biochemistry, Biochemistry Department
University of Wisconsin, Madison
Characterization of yeast ribonucleic acids

EMPLOYMENT HISTORY

1969-71 National Institutes of Health Postdoctoral Fellow
Dept. of Molecular Biology, University of California, Berkeley, CA.
Studies of the control of RNA synthesis in bacteria, using chemical and genetic techniques. Terminal nucleotide analysis of viral RNAs.

1971-73 Research Associate, Division of Biological and Medical Sciences,
Brown University, Providence, RI.
Biochemistry of schistosomiasis.
Isolation of enzymatically and serologically-active materials.

1974-89 Research Chemist (and Division Chief during 1983-1985)
Bacteriology Division,
U.S. Army Medical Research Institute of Infectious Diseases,
Frederick, MD.
Characterization of bacterial toxins.

1989-2001 Research Chemist, Oral Infection and Immunity Branch
National Institute of Dental and Craniofacial Research
National Institutes of Health, Bethesda, MD.
Structure and function of bacterial protein toxins.

2001-present Senior Investigator
Chief, Bacterial Toxins and Therapeutics Section
National Institute of Allergy and Infectious Diseases
National Institutes of Health, Bethesda, MD.
Structure and function of bacterial protein toxins.

MEMBERSHIPS IN PROFESSIONAL SOCIETIES

1974-present American Society for Microbiology
1985-present American Academy of Microbiology

OTHER ACTIVITIES

1980-1987	Member, Editorial Board, Infection and Immunity
1987-1991	Editor, Infection and Immunity
1995-1998	Member, NIH Institutional Biosafety Committee
1998-	Member, NIAID Working Group on Anthrax Vaccines
2000-	Member, Working Group on Recombinant Toxins, NIH Office of Biotechnology Activities, OD, NIH

Publications:

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Borohydride reduction of periodate-oxidized chain ends (RNA).
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2. Lewandowski, L. J., J. Content, and **S. H. Leppla**.
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3. Lewandowski, L. J. and **S. H. Leppla**.
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4. **Leppla, S. H.**
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10. Walker, H. L., C. G. McLeod, Jr., **S. H. Leppla**, and A. D. Mason, Jr.
Evaluation of *Pseudomonas aeruginosa* toxin A in experimental rat burn wound sepsis.
Infect. Immun. 25:828-830. 1979.

11. Dorland, R. B., J. L. Middlebrook, and **S. H. Leppla**.
Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells.
J. Biol. Chem. 254:11337-11342. 1979.

12. **Leppla, S. H.**, R. B. Dorland, and J. L. Middlebrook.
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13. Brown, J. E., M. A. Ussery, **S. H. Leppla**, and S. W. Rothman.
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14. Middlebrook, J. L., R. B. Dorland, **S. H. Leppla**, and J. D. White.
Receptor-mediated binding and internalization of *Pseudomonas* exotoxin A and diphtheria exotoxin by mammalian cells.
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15. **Leppla, S. H.** and R. B. Dorland.
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18. **Leppla, S. H.**
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19. Vodkin, M. H. and **S. H. Leppla**.
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21. **Leppla, S. H.**
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Cloning and characterization of a gene whose product is a *trans*-activator of anthrax toxin synthesis.
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Structure-function analysis of *Bacillus anthracis* edema factor by using monoclonal antibodies.
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Anti-tumor efficacy of a urokinase activation dependent anthrax toxin*

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Abbreviations:

CMG2	Capillary morphogenesis gene 2
EF	Oedema factor
FP59	Fusion protein 59
LF	Lethal factor
LLC	Lewis lung carcinoma
PAI-1	Plasminogen activator inhibitor 1
PrAg	Protective antigen
TEM8	Tumor endothelial marker 8
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

Abstract

Previously, we have generated a potent pro-drug consisting of modified anthrax toxins that is activated by urokinase plasminogen activator (uPA). The cytotoxicity of the drug, PrAg-U2 + FP59, is dependent on the presence of receptor-associated uPA-activity. Local intradermal administration of PrAg-U2 + FP59 adjacent to the tumor nodules in mice with transplanted solid tumors had a potent anti-tumor effect. In succession of these experiments we have now investigated the systemic anti-tumor efficacy of PrAg-U2 + FP59.

C57Bl/6J mice bearing syngenic tumors derived from B16 melanoma, T241 fibrosarcoma, or Lewis Lung Carcinoma cells were treated with different mass ratios and doses of PrAg-U2 + FP59. Tumor volumes were recorded daily by caliper measurements. In some experiments dexamethasone was co-administered.

Our data show a significant anti-tumor effect of systemic administration of PrAg-U2 + FP59 in three syngenic tumor models. Optimal anti-tumor effect and low toxicity was obtained with a 25:1 mass ratio between the two components (PrAg-U2:FP59). The experiments show that PrAg-U2 + FP59 displays a clear dose-response relationship with regard to both anti-tumor efficacy and systemic toxicity. Dose-limiting toxicity appeared to be due to activation of the pro-drug by uPA-uPAR in the intestinal mucosa. Concurrent treatment with dexamethasone was found to prevent dose-limiting toxicity.

Taken together these data indicates that uPA-activated toxins may be promising candidates for targeted therapy of human cancers that over-express uPA and its receptor.

Introduction

Urokinase plasminogen activator (uPA) and its cellular receptor (uPAR) are part of a proteolytic system associated with degradation and remodelling of the extracellular matrix in cancer, a hallmark of malignant transformation (1-4). The serine protease uPA is secreted as an inactive pro-enzyme (pro-uPA) that after binding to uPAR can be converted to active cell-surface associated uPA. uPA in turn activates plasminogen to plasmin. The membrane assembly of the plasminogen activation (PA) system components is required to favour and confine plasminogen activation in proximity of the cell surface (5,6). uPA and uPAR are overexpressed in many types of human cancers, such as ductal breast carcinomas, colon adenocarcinomas, and squamous cell carcinomas in head and neck, lung, skin and oesophagus (2,7). The fact that both uPA and uPAR are over-expressed in cancer prompted the development of cytotoxic pro-drugs that are specifically activated by cell surface bound uPA (8,9).

Native anthrax toxin, secreted by the Gram-positive bacterium *Bacillus anthracis,* consists of three individually non-toxic protein components: protective antigen (PrAg), lethal factor (LF), and oedema factor (EF). PrAg binds to the specific cellular receptors, tumor endothelial marker 8 (TEM8) or capillary morphogenesis gene 2 (CMG2), and is subsequently cleaved by cell surface associated furin or furin-like proteases, leading to the dissociation of an amino-terminal fragment (10-14). The carboxy-terminal part of PrAg remains bound and self-associates to form a heptamer. The heptamer structure enables binding of LF and/or EF which result in insertion of the heptamer in endosomal membranes and subsequent translocation of LF and/or EF into the cytosol where they cause cytotoxicity (15-18). The obligatory requirement for proteolytic processing of PrAg at the cell surface provides a way to re-engineer the toxin for activation by other cell surface associated enzymes than furin. Based on the availability of phage display-derived synthetic uPA-cleavable sequences (19) we generated a uPA activation dependent form of the anthrax toxin protective antigen (PrAg-U2) in which the native furin cleavage site was replaced by a uPA cleavage site (8). To increase the cytotoxicity of LF, a fusion protein (fusion protein 59 [FP59]), consisting of residue 1-254 of LF and the catalytic domain of *Pseudomonas* exotoxin A, was constructed (20). The catalytic domain of *pseudomonas* exotoxin A consists of a ADP-ribosylation domain that catalyzes the covalent attachment of ADP to elongation factor 2 thereby blocking protein synthesis. Administration of PrAg-U2 together with FP59 constitutes a potent cytotoxic pro-drug requiring activation by uPA. Thus, cells displaying active cell surface bound uPA and expressing either TEM8 or CMG2 are killed by the drug due to a block of protein synthesis.

uPA and uPAR are either not expressed or only one of the components are expressed at low levels in normal tissues. The expression of both is readily induced during normal tissue remodelling processes, such as wound healing, mammary gland involution and trophoblast invasion (1,21,22). Despite the generally low expression of uPA and uPAR in normal tissues, toxicity in T cell areas of the spleen and lymph nodes, bone marrow, adrenal cortex, and osteoblasts following administration of PrAg-U2 + FP59 was observed in a previous study (9).

Both *in vitro* and *in vivo* it has been demonstrated previously that activation and cytotoxicity of co-administered PrAg-U2 + FP59 are dependent on the presence of active uPA on the cell surface. In culture, uPAR negative cells were found to be insensitive to the reengineered toxin and uPA negative cells only were sensitive when uPA was added to the medium. In addition, cultured cells could be rescued from PrAg-U2 + FP59-induced death by addition of blockers of the interaction between uPA and its receptor (8). Furthermore, PrAg-U2 showed cell-surface uPA-dependent toxin activation *in vivo*, as revealed by the complete lack of toxic effects in mice deficient in plasminogen, uPA, or uPAR at doses that were lethal to wild-type mice, indicating that the component of the plasminogen activator system are essential for activation of the toxin. In the same study, FP59 or FP59 in combination with a non-cleavable form of PrAg was tolerated well by mice even when administered in large doses (200 μg). Moreover, LLC tumors grown in plasminogen knockouts mice were unresponsive to treatment with the re-engineered toxin, confirming that an intact plasminogen activator system is required in order for the toxin to become activated. Furthermore, local intradermal injections of PrAg-U2 + FP59 beneath transplanted solid tumors showed a significant anti-tumor effect in three different murine cancers (9).

In succession of these studies, we have now documented the anti-tumor effect of PrAg-U2 + FP59 after systemic administration and have defined the dose-relationship between the anti-tumor effects and the toxicity of the pro-drug. Moreover, we have found that co-administration of dexamethasone dramatically increases the therapeutic window of the uPA activated toxin.

Materials and Methods

Tumor transplantation and Treatment

Lewis lung carcinoma (LLC) cells and B16 melanoma cells were grown in DMEM with 10% FBS. T241 fibrosarcoma cells were grown in DMEM with 1% glutamax/1% nonessential amino acid mix/10% FBS. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The cells (10^6 cells/mouse) were injected subcutaneously in the right flank of C57Bl/6J mice. When the

tumors reached a volume of approximately 50 mm^3, the mice were allocated in treatment arms of 7-13 mice with equal median tumor sizes, and the treatment was started (day 0). The mice were given intraperitoneal (i. p.) injections of PBS or PrAg-U2 + FP59 in PBS. The construction and purification of both PrAg-U2 and FP59 has previously been described (8,20). The mice were treated with three day intervals, starting at day 0. In one experiment, concurrent daily treatment with 5 mg/kg dexamethasone (Dexamethasone VETRANAL, Sigma-Aldrich, Copenhagen, Denmark) was carried out. The experiments were terminated at day 9 with the exception of experiment 4.

Animals

Institutional guidelines for animal welfare and experimental conduct were followed in all experiments. All mice (5-8 weeks old males) were obtained from Taconic M & B (Ry, Denmark). The mice were conditioned to the new environment for one week before starting the experiment. The mice were shaved before tumor cell implantation to facilitate size measurements of the tumors. All mice were ear-tagged to ensure identification of each individual mouse. This was done while the mice were anaesthetized by subcutaneous injections of ketamine (10 mg/kg) and xylazine (1 mg/kg) in isotonic 0.9 % NaCl solution. The mice were euthanized by cervical dislocation at the end of each experiment.

Tumor growth analysis

The sizes of the tumors were determined by daily caliper measurements of two orthogonal diameters in mm during the growth phase. The sizes were calculated by the following empirical formula (23):

$$\text{Tumor size} = d_1 \times d_2^{3/2} \times \pi/6 \times K$$

d1 and d2 are orthogonal diameters in mm (adjusted for skin thickness by subtracting 0.5 mm from the measurement)
K is an empirical constant = 0.67

Growth inhibitory effects were evaluated by comparison of tumor sizes (by two a tailed student's t-test) and by Kaplan-Meier log rank analysis. A value of $p<0.05$ was considered statistically significant.

Evaluation of toxicity

For each treatment dose the number of dead mice was recorded and the fractional occurrence of toxic death was expressed by a lethality index, calculated by the following formula:

$$\text{Lethality index} = \sum(10\text{-dtx})/n \times 10$$

10 is the number of days of the experiment
dtx is the day of event (death of a mouse)
n is the number of mice in the treatment arm

The lethality index is the fraction of measurements lost due to toxicity relative to the total number of measurements, and therefore includes information not only on the number of mice dead in each treatment groups, but also on when the mice had died during the experiment. Further, the weight of the mice was recorded on a daily basis.

Histological analysis

For histological analysis intestines, lung, heart, liver, kidney, and spleen were dissected from B16 melanoma-bearing mice treated with 1.96 mg/kg PrAg-U2 + 0.052 mg/kg FP59 or PBS i. p. three times at three day intervals. The tissues were fixed overnight in 4% paraformaldehyde in PBS and embedded in paraffin. Five µm sections were deparaffinated, rehydrated, and stained with hematoxylin/eosin.

Cellular cytotoxicity assay

40,000 cells were cultured overnight in growth medium in 96-well plates. The cells were treated with PrAg-U2 (0-3000 ng/ml) combined with FP59 (50 ng/ml) for 24 hours. Cell viability was assayed by adding 40 µl of 5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich, Copenhagen, Denmark) at 48 hours. The cells were incubated with MTT for one hour; the medium was removed, and the purple pigment produced by the cells was dissolved in 200 µl DMSO. Absorbance was measured at A_{570}. Cell viability (% of control) was plotted versus $\log_{10}$ PrAg-U2 concentration.

Results

Significant anti-tumor effect of PrAg-U2 + FP59 administered systemically

We first assayed the anti-tumor effect of the uPA-activated toxin after intraperitoneal administration to Lewis Lung Carcinoma (LLC) transplanted mice. Mice bearing solid subcutaneous tumors of

approximately 50 mm^3 volumes were allocated to treatment arms with different doses of PrAg-U2 + FP59 three times with three days intervals (day 0, 3 and 6). These mice were treated with different absolute doses, but all with a mass ratio of PrAg-U2 to FP59 of 3:1, in accordance with the ratio previously applied as local, intradermal/peritumoral injections (9). The lowest dose (0.2 mg/kg PrAg-U2 + 0.068 mg/kg) resulted in significant anti-tumor effect ($P<0.05$), whereas the higher doses were lethal (Table I, experiment 1).

Comparison of different ratios of PrAg-U2:FP59

Anthrax receptors are ubiquitously expressed in virtually all types of mammalian cells, which implies that a relatively large amount of PrAg-U2 protein is necessary in order to saturate these binding sites. The efficiency of this toxin system may therefore be dependent on the ratio between the two components PrAg-U2 and FP59. To investigate this and approximate the optimal ratio between the two components, we compared the anti-tumor effect of three different doses of the toxin with PrAg-U2:FP59 ratios of 3:1, 10:1, and 25:1 by treating LLC-bearing mice with 0.2 mg/kg PrAg-U2 + 0.068 mg/kg FP59, 0.68 mg/kg PrAg-U2 + 0.068 mg/kg FP59, 1.70 mg/kg PrAg-U2 + 0.068 mg/kg FP59. The most pronounced anti-tumor effect was obtained with the 25:1 ratio between the two components (Figure 1 and Table I, experiment 2).

Using 25:1 to 50:1 ratios we next tested the effect of systemically applied toxin in mice bearing T241 fibrosarcomas or B16 melanomas. In the experiment with T241-bearing mice, three different doses of PrAg-U2 + FP59 were applied, two doses with a ratio of 25:1 of PrAg-U2 to FP59 (0.85 mg/kg PrAg-U2 + 0.034 mg/kg FP59 and 1.70 mg/kg PrAg-U2 + 0.068 mg/kg), and one dose of PrAg-U2 + FP59 with a ratio of PrAg-U2 to FP59 of 50:1 (2.00 mg/kg + 0.040 mg/kg). The highest dose with a mass ratio 25:1 and the dose with a ratio of 50:1 both resulted in significant tumor growth inhibition (Figure 2 and Table I, experiment 3). In the experiment with B16-bearing mice, three ratios of PrAg-U2 + FP59 were applied. FP59 was held at a constant dose (0.052 mg/kg) and the doses of PrAg-U2 were increased to give ratios of 25:1, 37.5:1 and 50:1. All these doses resulted in significant tumor growth inhibition of the B16 melanomas (Table I, experiment 4).

To investigate if prolonged treatment of the mice could further inhibit the tumor growth, we decided to extent the treatment period. LLC-bearing mice were treated with 1.00 mg/kg PrAg-U2 + 0.04 mg/kg FP59) day 0, 3, 6, 12, and 15. The treatment resulted in a significant anti-tumor effect: however, the extended treatment did not suppress tumor growth completely, indicating that not all uPAR positive tumor cells were killed by the treatment (Figure 3 and Table I, experiment 5)

Taken together these results demonstrate that PrAg-U2 + FP59 is active when administered systemically and that the pro-drug induces a significant growth delay in LLC, T241 and B16 transplanted tumors.

Dose-limiting toxicity after systemic treatment

In the experiments described above we observed a dose-limiting toxicity with the highest doses. As a measure of this toxicity we calculated a lethality index, which reflects not only how many of the mice that had died but also how early they had died (see materials and methods). To investigate if increasing the dose of either of the two components led to an increase in lethality in B16-bearing mice, we first kept the dose of FP59 at a constant level of 0.052 mg/kg, while the dose of PrAg-U2 was varied from 1.30 mg/kg, 1.96 mg/kg, or 2.61 mg/kg (Table I, experiment 4). The doses resulted in lethality indexes of 13%, 22%, and 36%, respectively, demonstrating that increasing the dose of PrAg-U2, when FP59 is kept at a constant level, results in an increase in lethality. Analogous to what is observed by increasing PrAg-U2 the lethality was increased from 11% to 50% in B16-bearing mice, when the dose of FP59 was increased from 0.048 mg/kg to 0.144 mg/kg and PrAg-U2 was held constant at 1.20 mg/kg (Table I, experiment 6).

All together seven different ratios of PrAg-U2 to FP59 were tested in the experiments, ranging from 3:1 to 50:1. To analyze the relationship between doses, ratios of the two components, and lethality, we plotted PrAg-U2 doses against the doses of FP59 for each ratio (Figure 4). For all the ratios tested, this plot shows that an increase in dose results in an increase of the lethality index.

Gross inspection of mice treated with the highest doses of PrAg-U2 + FP59 revealed ulcerations of the anal region and oedema of the small intestines (Figure 5B). No other gross pathology was noted in any mice as a result of the treatment. Histological investigation of intestines of mice treated with either vehicle or 1.96 mg/kg PrAg-U2 + 0.052 mg/kg FP59 revealed that the intestines of the toxin-treated mice were clearly affected by the treatment. The villi in the small intestine were severely inflamed, the tips of the villi were necrotic, and occasionally necrotic tissue was shed into the intestinal lumen (Figure 5D). The villi of the vehicle-treated animals appeared normal. No histological signs of toxicity were observed in the liver, lung, kidneys, heart, and spleen of the toxin-treated B16 melanoma-bearing mice (data not shown).

Dose-limiting toxicity is efficiently inhibited by dexamethasone

To investigate if supplying the mice with an anti-inflammatory drug could attenuate the dose-limiting toxicity, daily treatment with the glucocorticoid dexamethasone was initiated. B16-bearing mice were treated with 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59, resulting in a lethality index of 20%. Concurrent treatment with dexamethasone reduced the lethality index to 1% (Figure 6 and Table I, experiment 7). In the same experiment we recorded the weight of the mice on a daily basis. Mice treated with 2.40 mg/kg PrAg-U2 + 0.048 mg/kg had a significant weight loss compared to PBS treated mice ($P<0.05$). Interestingly, when the mice received dexamethasone in addition to PrAg-U2 +FP59 no weight loss was observed (Figure 7). Since dexamethasone has previously been reported to have growth inhibitory effects in B16 melanoma tumors (24), we specifically addressed this potential bias. Daily dexamethasone treatment of 5 mg/kg for 9 days did not induce significant growth delay of B16 melanomas.

Direct killing of tumor cells by PrAg-U2 + FP59

In order to clarify whether the anti-tumor effect of PrAg-U2 + FP59 observed *in vivo* is caused by direct killing of the tumor cells or by affecting the stroma, we analysed the cytotoxic effect of the toxin *in vitro* by a colorimetric cytotoxicity assay. All three murine cell lines were sensitive to PrAg-U2 + FP59 treatment (data not shown) in a dose-dependent manner, demonstrating that the cells express the component required for activation and intoxication of the cells. Furthermore, the results indicate that the *in vivo* anti-tumor effect is mediated at least in part by a direct effect on the tumor cells.

Discussion

The well-documented over-expression of uPA and uPAR in several types of cancer makes the plasminogen activation-system an attractive candidate for targeted therapy. In many experimental *in vivo* studies, the aim has been to block the plasminogen activation -system either with inhibitors of the active site of uPA (25-27), or with inhibitors of the uPA/uPAR interaction (for review see (7)). In the present approach we have utilized a two-component pro-drug system, PrAg-U2 + FP59, which consists of modified anthrax toxins that specifically kill cells dependent on the presence of cell-surface associated uPA-activity, resulting in a direct cytotoxic effect both *in vitro* and *in vivo* (8,9).

In our previous *in vivo* studies, local administration of the pro-drug resulted in a substantial anti-tumor effect and in some cases tumor eradication. PrAg-U2 + FP59 was injected intradermally in the area surrounding the tumor nodules in order to obtain high local concentrations of drug in and

around the tumors (9). Although local administration of PrAg-U2 + FP59 in clinical settings may be feasible and even desirable in special types of malignancies such as head and neck cancer or brain cancer, most cancer-treatment is systemic. We have therefore investigated the anti-tumor activity of PrAg-U2 + FP59 after systemic administration. Importantly, we find that the modified toxins have significant anti-tumor effects after intraperitoneal administration. The optimal ratio between PrAg-U2 and FP59 for systemic use is 25:1 instead of the 3:1 ratio used for local administration. The reason for the need of a larger excess of PrAg-U2 to FP59 for systemic administration is probably the existence of a "sink-effect" due to the ubiquitous expression of PrAg receptors in the normal tissues, particularly CMG2 and to some extent TEM8 (10,28). The anti-tumor effect of systemically administered drug was clearly dose-dependent. However, high doses of PrAg-U2 + FP59 also elicited toxicity. By dose-optimization, we could approximate the optimal treatment dose that shows a significant growth inhibitory effect and at the same time low toxicity. However, a definitive determination of the therapeutic window of the pro-drug is complicated by the two-component nature of the drug, and thus could not be defined by an absolute or single value since it depends on the dose of each of the two components. If a low dose of FP59 is administered a high dose of PrAg-U2 can be administered without toxicity and vice versa.

PrAg-U2 + FP59 targets cells that harbours receptor-bound uPA on their surface. In some types of cancer, uPA and uPAR are often expressed by the stromal cells and only in a subpopulation of the cancer cells, which means that the anti-tumor effect of PrAg-U2 + FP59 in such cases may be obtained either by direct killing of cancer cells, or indirectly by destruction of the cancer-associated stroma. In colon and breast cancer, uPAR is predominantly expressed by macrophages, whereas uPA is produced and secreted mainly from myofibroblasts (29-33). However, uPAR is also expressed by budding cancer cells in colon cancer (34) and may also be expressed at a certain level in some breast cancer cells (30,35). This expression pattern is clearly distinct from that observed in squamous cell carcinomas, in which the predominant expression of uPA and uPAR is seen in the cancer cells as typified by squamous cell cancer of the skin (36) and esophagus (37). Lung, head and neck, cervical and esophageal squamous cell carcinomas may thus be good candidates for PrAg-U2 + FP59 based therapy aiming at targeting the cancer cells directly.

The systemic toxicity of uPA activated toxin may be related to an inflammatory response elicited by PrAg-U2 + FP59. We therefore anticipated that steroid treatment with dexamethasone would alleviate toxicity. In addition to the anti-inflammatory effect, dexamethasone has been reported to decrease the level of uPAR expression (38). Co-administration of dexamethasone with a high dose

of PrAg-U2 + FP59 completely eliminated toxicity compared with the group that received PrAg-U2 + FP59 alone. Whether dexamethasone abrogates a general systemic inflammatory condition or a local tissue specific damage in the PrAg-U2 + FP59 treated mice needs to be investigated in more detail.

We have observed both gross and histological signs of intestinal affection in PrAg-U2 + FP59 treated mice that were clearly suffering from malaise. The finding of strong inflammation and necrosis in the tip of the intestinal villi indicate that this part of the villi is especially vulnerable to the toxin. The reason for this may be that both uPA and uPAR are expressed in the villi in the normal intestine. uPAR is expressed in the epithelial mucosa cells lining the villi and uPA is expressed in fibroblast-like cells located just beneath the epithelium, and may hence be found at the distal epithelium (39-41). In addition, CMG2 and TEM8 is expressed in the small intestine (10,42,43). The co-localised expression of the components required for activation of the pro-drug may thus explain the specific damage to the epithelial cells at the tip of the villi. In support of this hypothesis, our previous studies showed that PAI-1-deficient mice presented oedema and hemorrhagia of the small intestine even at very low PrAg-U2 + FP59 doses (9).

The mechanism of *in vivo* anti-tumor effect of PrAg-U2 + FP59 is still not clearly understood and need further analysis. Evidence from ours previous studies and studies by others (9,44) points both at a direct cytotoxic effect of PrAg-U2 + FP59 towards tumor cells and also at an anti-angiogenic effect. We observed pronounced tumor vascular damage with angiectasis, vascular stasis, and hemorrhagia in PrAg-U2 + FP59 treated Lewis Lung Carcinomas (9). However, the endothelial damage might be secondary to tumor cell cytotoxicity since the anti-tumor effect of PrAg-U2 + FP59 in Lewis Lung Carcinoma transplanted uPAR$^{-/-}$ mice was equal to the effect seen in wild type mice (9). Histological investigations of tumor tissue from PrAg-U2 + FP59 treated Lewis Lung Carcinoma bearing mice, previously performed, showed cytoplasmatic shrinkage, nuclear condensation, and cessation of BrdU incorporation into the tumor cells. In addition, TUNEL staining revealed no increase in tumor cell apoptosis, suggesting that the tumor cells were undergoing necrotic cell death and not apoptosis. Furthermore, we have now demonstrated that PrAg-U2 + FP59 is capable of killing Lewis Lung Carcinoma, B16 melanoma and T241 fibrosarcoma cells *in vitro* by cytotoxicity experiments. It has previously been shown that Lewis Lung Carcinomas express both uPA (45) and uPAR (46). Taken together, these data strongly suggest that the anti-tumor effect is mediated, at least in part, by direct killing of the tumor cells.

In conclusion, we here provide evidence of potent anti-tumor effect after systemic administration of an uPA-activated toxin, and furthermore the possibility to block the dose-limiting toxicity by co-administration of dexamethasone. These findings warrant further development of uPA-activated toxins in the clinic for specific targeting of cancers with over-expression of uPA and uPAR.

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Table I. Anti-tumor effect and toxicity of PrAg-U2 + FP59.

Experiment number (tumor type)	PrAg-U2 (mg/kg)	FP59 (mg/kg)	Ratio	Dexamethasone (5 mg/kg)	Lethality index[§]	Number of Mice	t-test (p-value)	Kaplan Meier (p-value)	Number of dead mice	Day of death
1 (LLC)	0.20	0.068	3:1	-	8%	10	0.0195	0.0117	2	5 and 7
	0.60	0.200	3:1	-	79%	10	-	-	10	1, 1, 1, 2, 2, 2, 3, 3, 3, and 3
	1.20	0.400	3:1	-	80%	10	-	-	10	1, 1, 1, 2, 2, 2, 2, 3, 3, and 3
	3.60	1.200	3:1	-	82%	10	-	-	10	1, 1, 1, 1, 1, 2, 2, 3, 3, and 3
2 (LLC) See Figure 1	0.20	0.068	3:1	-	0%	12	0.0004	NS	0	-
	0.68	0.068	10:1	-	3%	11	0.0005	0.0102	1	7
	1.70	0.068	25:1	-	20%	12	0.0001	0.0035	4	2, 4, 4, and 6
3 (T241) See Figure 2	0.85	0.034	25:1	-	0%	12	0.0360	NS	0	-
	2.00	0.040	50:1	-	6%	13	0.0022	0.0002	2	5 and 7
	1.70	0.068	25:1	-	23%	12	0.0060	0.0005	6	4, 4, 5, 6, 7, and 7
4 (B16)	1.30	0.052	25:1	-	13%	10	0.0002	0.0208	2	2 and 5
	1.96	0.052	37.5:1	-	22%	10	0.0246	0.0001	4	3, 5, 5, and 5
	2.61	0.052	50:1	-	36%	10	0.0043	0.0001	8	4, 5, 5, 5, 5, 5, 6, and 9
5* (LLC) See Figure 3	1.00	0.040	25:1	-	-	10	0.007	0.003	3	8, 11, and 14
6 (B16)	1.20	0.048	25:1	-	11%	8	NS	NS	4	5, 5, 8, and 9
	1.20	0.096	12.5:1	-	11%	8	0.0303	NS	3	6, 7, and 8
	1.20	0.144	8.3:1	-	50%	8	-	-	8	4, 4, 5, 5, 5, 5, 5, 5, and 7
7 (B16) See Figure 6	-	-	-	+	0%	7	NS	NS	0	-
	2.40	0.048	50:1	-	20%	8	0.0255	0.0083	5	6, 6, 6, 7, and 9
	2.40	0.048	50:1	+	1%	9	0.0074	0.0079	1	9

[§]As defined in Materials and Methods.

*The mice were treated at day 0, 3, 6, 9, 12, and 15 as opposed to day 0, 3, and 6.

NS: not significant

Figure legends

Figure 1

The anti-tumor effect is dependent on the PrAg-U2:FP59 ratio. Upper panel: LLC bearing mice were treated with PBS (–●–), 0.2 mg/kg PrAg-U2 + 0.068 mg/kg FP59 (–○–), 0.68 mg/kg PrAg-U2 + 0.068 mg/kg FP59 (–▼–), or 1.7 mg/kg PrAg-U2 + 0.068 mg/kg (–▽–), giving ratios of PrAg-U2:FP59 of 3:1, 10:1, and 25:1, respectively. The most pronounced anti-tumor effect was observed with the ratio of 25:1, indicating that the effect is dependent on the ratio of the two components. The sizes of the tumors are expressed as mean tumor size in mm^3 ± SEM. Lower panel: The number of remaining mice in each treatment group is showed in the lower panel of the figure. The symbols correspond to the symbols used in the upper panel of the figure.

Figure 2

Significant anti-tumor effect of PrAg-U2 + FP59 in T241 fibrosarcoma bearing mice. Upper panel: The mice were treated with PBS (–●–), 0.85 mg/kg PrAg-U2 + 0.034 mg/kg FP59 (–○–), 2.00 mg/kg + 0.040 mg/kg PrAg-U2 (–▼–), or 1.70 mg/kg PrAg-U2 + 0.068 mg/kg (–▽–) i. p. day 0, 3, and 6. The two highest doses of PrAg-U2 + FP59 resulted in significant anti-tumor effect, demonstrating the sensitivity of T241 fibrosarcoma towards PrAg-U2 + FP59 treatment. The sizes of the tumors are expressed as mean tumor size in mm^3 ± SEM. Lower panel: The number of remaining mice in each treatment group is showed in the lower panel of the figure. The symbols correspond to the symbols used in the upper panel of the figure.

Figure 3

Prolonged treatment. Upper Panel: LLC-bearing mice were treated with PBS (–●–), 1.00 mg/kg PrAg-U2 + 0.04 mg/kg FP59 (–○–) i. p. at day 0, 3, 6, 9, 12, and 15. The dose resulted in significant anti-tumor ffect, but the extended treatment could not completely inhibit the growth of the tumors. Lower panel: The number of remaining mice in the treatment group is showed in the lower panel of the figure. The symbol corresponds to the symbol used in the upper panel of the figure.

Figure 4

Lethality index is increased when the dose is increased. The lines represent different ratios of PrAg-U2:FP59 tested in C57Bl/6J mice by the three times three days interval dosage schedule, and the numbers refer to the lethality index induced by the dose. The figure illustrates that when keeping the ratio between the two components constant the toxicity increases when the dose of PrAg-U2 + FP59 is increased. Note: the two highest doses tested are not included in the figure.

Figure 5

PrAg-U2 + FP59-induced toxicity. Intestines of untreated C57Bl/6J mice (A) or mice treated with PrAg-U2 + FP59 (B). Small intestinal oedema was observed in mice treated with 2.4 mg/kg PrAg-U2 + 0.24 mg/kg FP59. Hematoxylin/eosin stained sections of small intestines from mice treated with PBS (C) or 1.96 mg/kg PrAg-U2 + 0.052 mg/kg FP59 (D). Note that the tip of the villi of the small intestine of the PrAg-U2 + FP59 treated mice are necrotic (arrows).

Figure 6

Protective effect of dexamethasone. Upper panel: B16 melanoma bearing mice were treated with PBS (–●–), dexamethasone (–○–), 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59 (–▼–), 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59 together with dexamethasone (–▽–) i. p. day 0, 3, and 6. Daily dexamethasone treatment itself did not result in significant growth delay in B16 melanoma tumors. The PrAg-U2 + FP59 treatment resulted in a significant anti-tumor effect. Co-administration of dexamethasone reduced the lethality index of 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59 from 20% to 1%, suggesting a protective function of dexamethasone. The sizes of the tumors are expressed as mean tumor size in $mm^3 \pm$ SEM. Lower panel:

Figure 7

Co-administration of dexamethasone attenuates PrAg-U2 + FP59 induced weight loss. The weight of mice treated with PBS (–●–), 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59 (–○–), 2.40 mg/kg PrAg-U2 + 0.048 mg/kg FP59 together with dexamethasone (–▼–) i. p. day 0, 3, and 6 was recorded daily. The mice treated with PrAg-U2 + FP59 alone had a significant weight loss compared to PBS treated mice, while in mice receiving PrAg-U2 + FP59 in combination with dexamethasone no weight loss was observed.

Figures

Figure 1

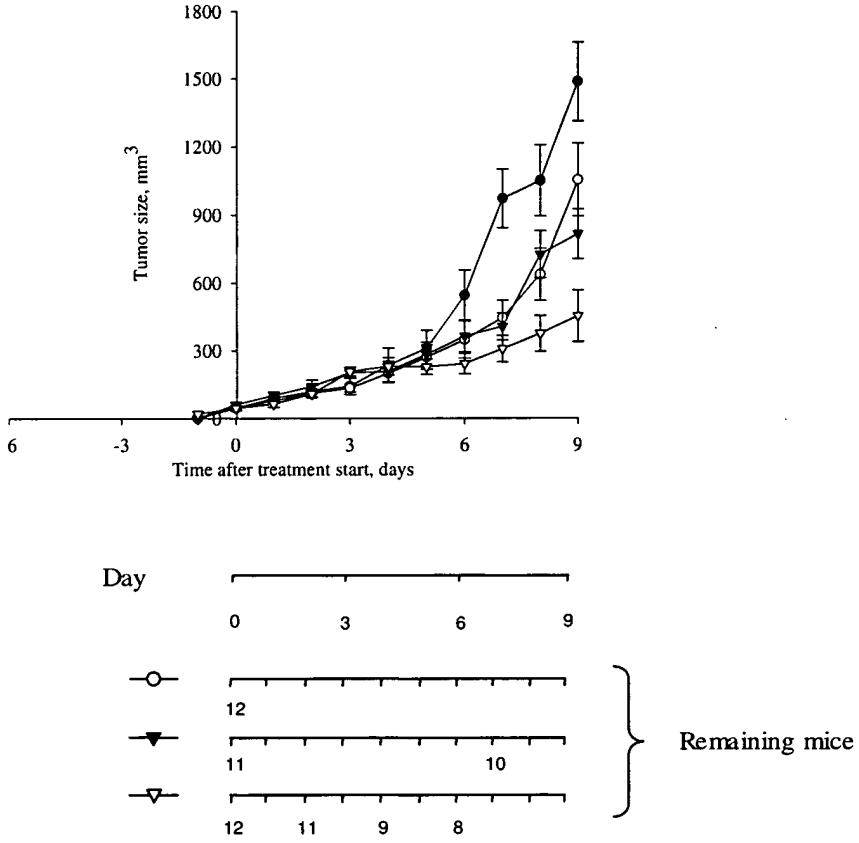


Figure 2

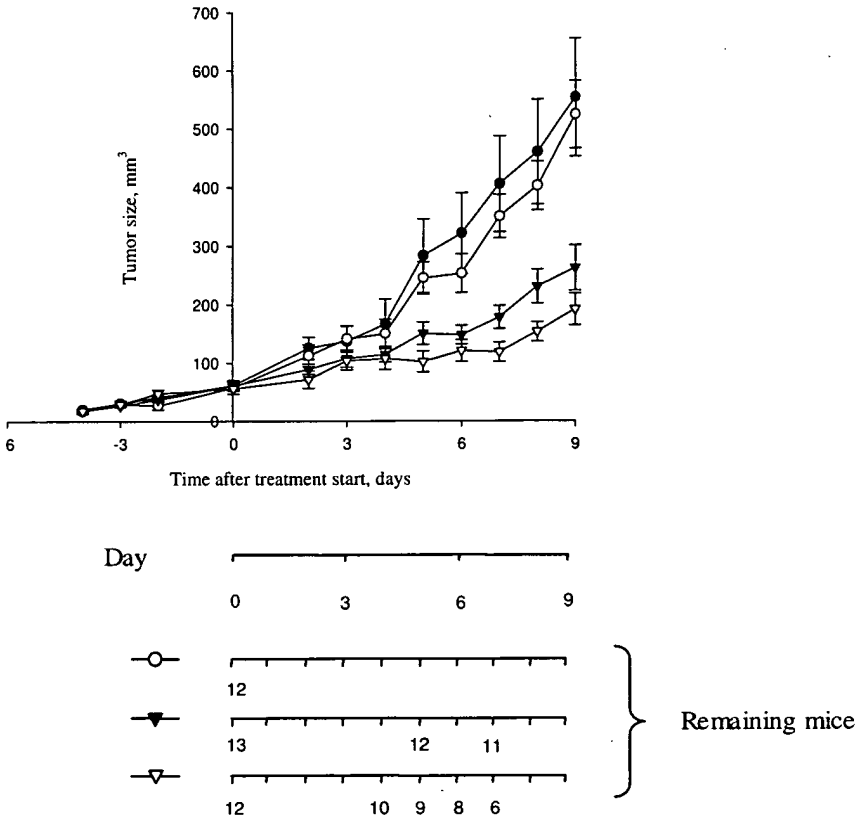

700
600
500
400
300
200
100
0
Tumor size, mm3
6
-3
0
3
6
9
Time after treatment start, days
Day
0
3
6
9
12
13
12
11
12
10
9
8
6
Remaining mice

Figure 3

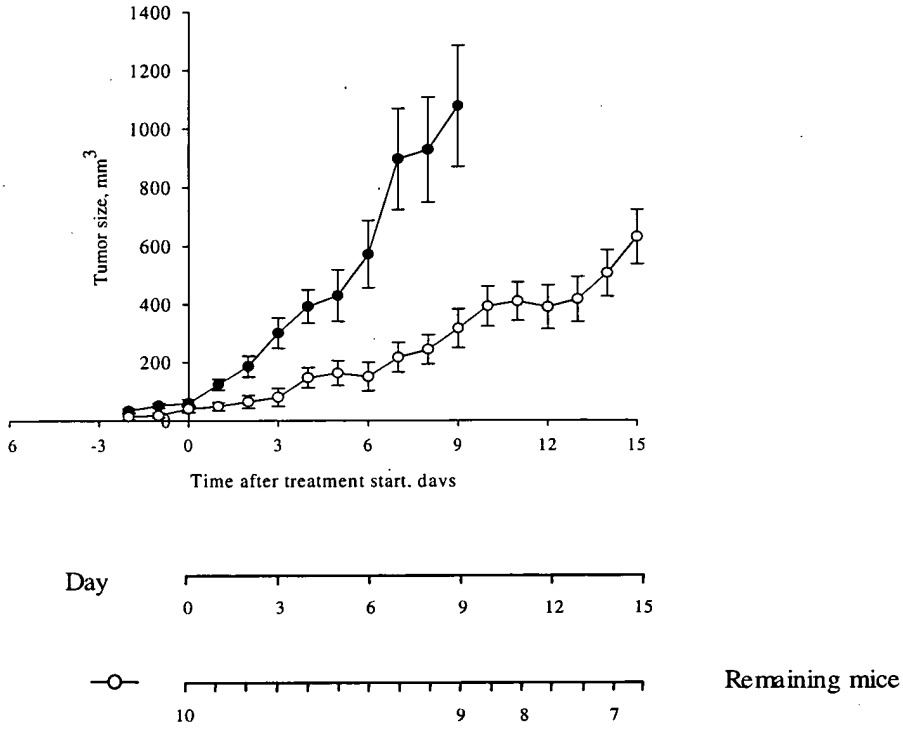

1400
1200
1000
800
600
400
200
0
Tumor size, mm3
6
-3
0
3
6
9
12
15
Time after treatment start, days
Day
0
3
6
9
12
15
10
9
8
7
Remaining mice

Figure 4

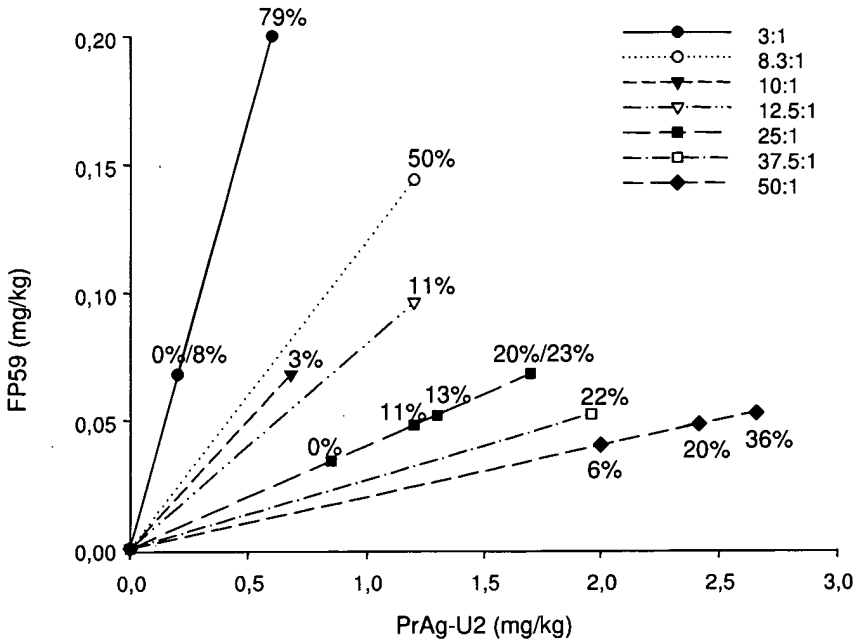

79%
50%
11%
0%/8%
3%
20%/23%
13%
11%
22%
0%
20%
36%
6%
3:1
8.3:1
10:1
12.5:1
25:1
37.5:1
50:1
0,20
0,15
0,10
0,05
0,00
FP59 (mg/kg)
0,0
0,5
1,0
1,5
2,0
2,5
3,0
PrAg-U2 (mg/kg)

Figure 5

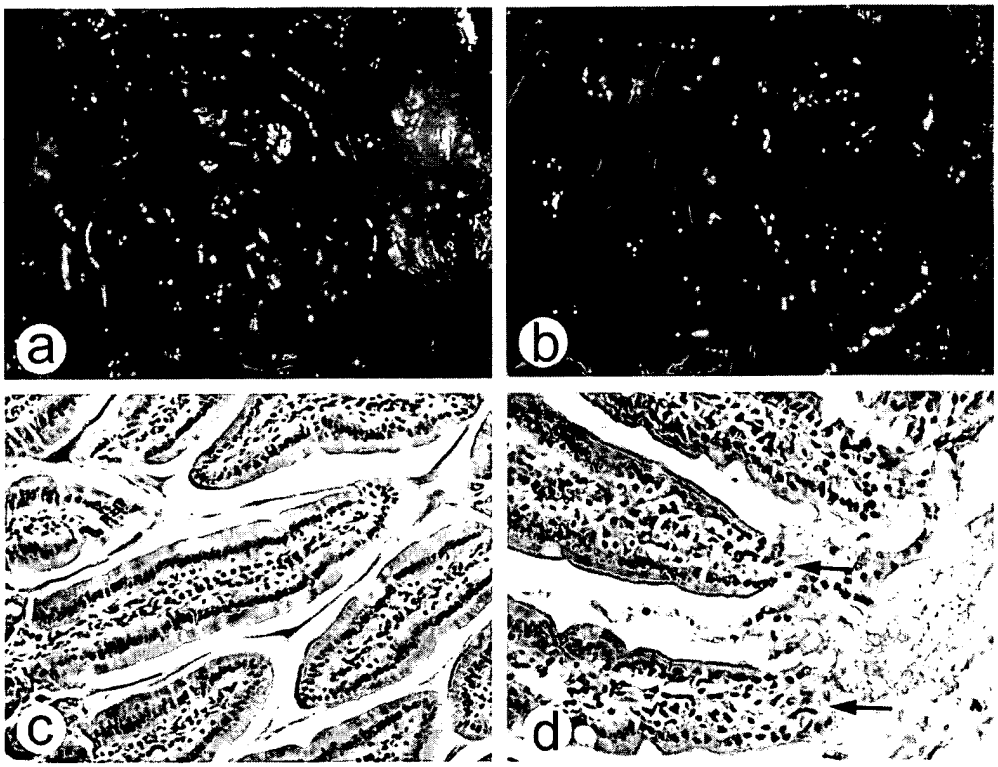
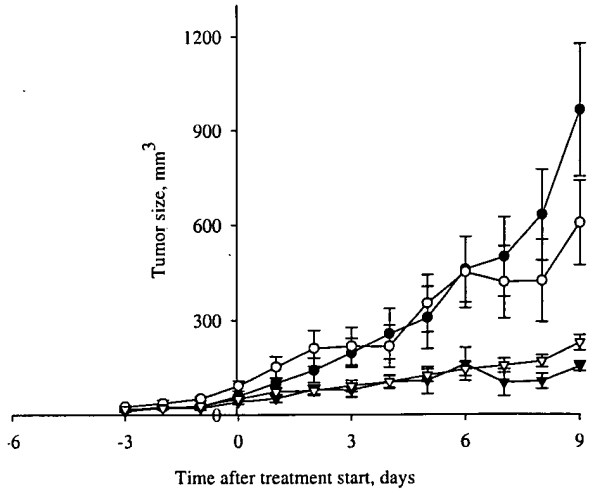


Figure 6


1200
900
600
300
0
Tumor size, mm3
-6
-3
0
3
6
9
Time after treatment start, days

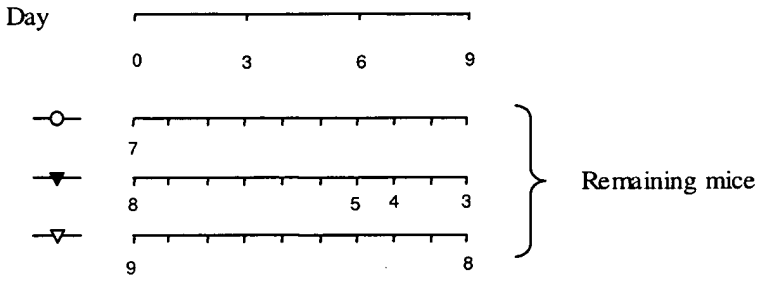
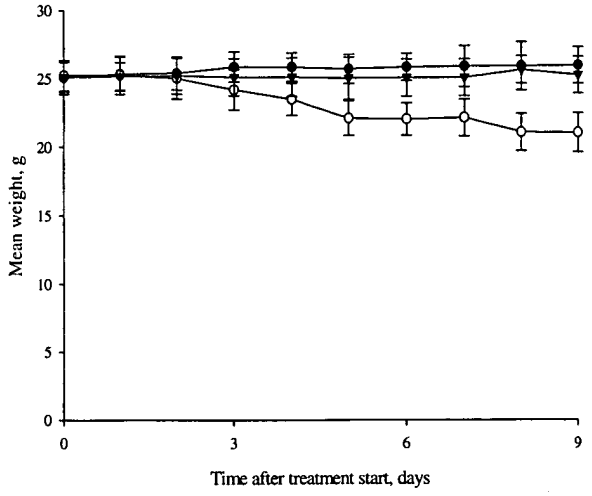

Day
0
3
6
9
7
8
5
4
3
9
8
Remaining mice

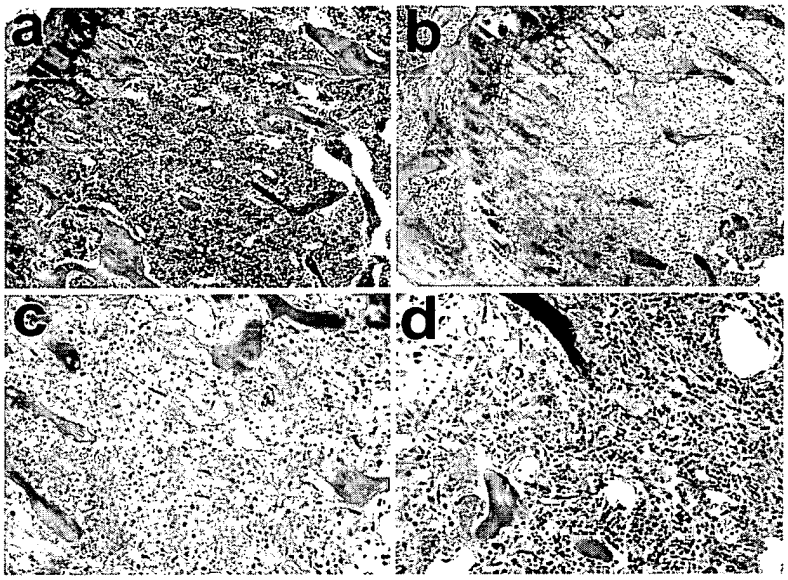
Figure 7


30
25
20
15
10
5
0
Mean weight, g
0
3
6
9
Time after treatment start, days

Supplementary Table 1. Properties and maximum tolerated doses of the PrAg proteins when injected intraperitoneally at days 0, 3, and 6

PrAg proteins or their combination	Proteolytic cleavage	Group L mutation Subsite II	Group R mutation		MTD3 (g) FP59=3 g
			Subsite I	Subsite III	
PrAg	Furin				0.25
PrAg-R200A	Furin	R200A			ND
PrAg-I210A	Furin			I210A	ND
PrAg-L1	MMP				4
PrAg-L1-R178A	MMP		R178A		ND
PrAg-L1-I210A	MMP			I210A	50
PrAg-L1-K214A	MMP			K214A	50
PrAg-U2	uPA				10
PrAg-U2-K197A	uPA	K197A			ND
PrAg-U2-R200A	uPA	R200A			100
PrAg-U2-R200A PrAg-L1-I210A	uPA MMP	R200A		I210A	30 15
PrAg-U2-R200A PrAg-L1-K214A	uPA MMP	R200A		K214A	30 15

ND: not done. PrAg-L1: previously characterized MMP-activated PrAg protein with furin site RKKR changed to MMP cleavage sequence GPLGMLSQ. PrAg-U2: previously characterized uPA-activated PrAg protein with furin site RKKR changed to uPA cleavage sequence PGSGRSA.



Supplementary Figure 1. Toxicity of the engineered PrAg proteins. Representative microscopic appearance of the bone and bone marrow (femur) at day 7 after treatment of the mice with PBS **(a)** or PrAg-U2-R200A/PrAg-L1-I210A **(b-d)** (45/22.5 μg, in the presence of 3 μg FP59) at day 0, 3, and 6. The bone marrow necrosis was observed in toxin-treated mice (**b**, H&E, 64×) but not in the mock-treated mice (**a**, H&E, 64×). At higher magnification, the necrotic area shows cellular debris and necrotic bone trabeculae (**c**); fibroblastic and vascular proliferations are seen in the periphery of damaged area representing ongoing repair processes (**d**).